REMARKS/ARGUMENTS

In response to the Office Action of August 30, 2005, Applicants have amended the claims, which, when considered with the following remarks, is deemed to place the present application in condition for allowance. Favorable consideration of all pending claims is respectfully requested.

The Examiner has acknowledged Applicants' election of claims in the response to the restriction requirement filed on May 10, 2005. Therefore, group II, claims 14-16 and 18 have been withdrawn from consideration by the Examiner. As presently amended, claim 14 recites a process for purifying on a large scale a product from a feedstock wherein the product is a rapamycin or a derivative thereof or an ascomycin or derivative thereof. Cyclosporin, which is not part of the elected subject matter, is no longer recited by this claim. Applicants therefore respectfully request that the Examiner reinstate claim 14. In addition, since claims 15 and 16 depend from presently amended claim 14, Applicants respectfully request that the Examiner also reinstate these two claims.

In the Office Action, the Examiner has stated that the Information Disclosure Statement (IDS) filed on 9/16/03 fails to comply with 37 CFR 1.98 (a)(1). The Examiner has therefore placed the IDS in the application but information referred to therein has not been considered. It is respectfully submitted that the IDS previously submitted contained all of the necessary information to identify the references cited by the Applicants so as to allow the Examiner to consider the art. Moreover, as of the submission date of the IDS, **September 16, 2003**, there was no requirement under 37 C.F.R. 1.98(a)(1) for "a column that provides a blank space next to each document to be considered, for the examiner's initials. *See* 37 C.F.R.§1.98 "[..."paras. (a) and (c) revised and par. (e) removed, 69

FR56481, Sept. 21, 2004, **effective Oct. 21, 2004**.] Thus, as of September 16, 2003, the submission date of the IDS in this application, the IDS was in compliance with 37 C.F.R. §1.98 in effect at the time.

Even if there was a requirement in place at the time for a blank space next to each document to be considered for the examiner's initials, the IDS contained ample space for the Examiner to initial each reference after the art was considered. Nonetheless, in order to expedite prosecution of the present application, a newly prepared IDS containing the same information as previously filed has been submitted herewith. It is not believed that any additional fee is required, but if an additional fee is required, please charge the same to Deposit Acct No. 04-1121.

The Examiner has objected to Claims 21 and 22 as allegedly substantially duplicative of claims 19 and 20. Claims 19-22 are presently canceled from the application. The objection to claims 21 and 22 is therefore moot.

Claims 11-13, 19 and 21 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. In particular, the position of the Examiner is that the phrase "a rapamycin or an ascomycin" is indefinite because use of the articles "a" and "an" make it appear that Applicants are intending to claim two genera of compounds. According to the Examiner however, it is unclear what members belong to the two genera. The Examiner has inquired how the genera differ from the compounds (i.e., a rapamycin and an ascomycin). For example, the Examiner has stated on page 4, paragraph 8 of the Office Action that the fourth paragraph on page 3 of Applicants' specification "merely lists these five compounds but does not describe the families." The

Examiner has also inquired how FK506 can be an ascomycin, noting that FK506 has a trans double bond in the macrolactone skeleton while ascomycin has a cis double bond at the same position.

As presently amended, claim 11 recites "or a derivative thereof" after each of the terms "rapamycin" and "ascomycin". The articles "a" and "an" as they appeared before "rapamycin" and "ascomycin" have been canceled from the claim. Support for these amendments may be found throughout the specification, e.g., page 3, lines 18-24. In addition, Applicants submit herewith as Exhibit A, Uchida, T. et al. (2002) "Identification of genes coding enzymes for ascomycin tetra-hydropyranose ring formation" *Internat. J. Mol. Med.* 9:141-145" that describes the relationship of the compounds recited in the presently amended claims. As described on page 141 of the paper, Ascomycin is a C-21 ethyl analog of FK506 and is a 23-member macrolide. Ascomycin is also known as immunomycin and FK520. *See* Figure 1. As may be seen from the dates of the references cited on page 141 of the paper, the relationship among ascomycin and derivatives thereof, and rapamycin and derivatives thereof, was known in the art at the time the present application was first filed. In view of the amendments to the claims and the foregoing remarks, withdrawal of the rejection of claims 11-13, 19, and 21 under 35 U.S.C.§ 112, second paragraph is respectfully requested.

Claims 17, 20 and 22 have been rejected under 35 U.S.C. § 112, second paragraph, because the limitation "FK506" is recited in the last line of each of these claims. It is the Examiner's position that no antecedent basis exists for this term. As stated above, Claim 11 has been amended to include the phrase "or a derivative therefore" after each of the terms "rapamycin" and "ascomycin." Since FK506 is a

derivative of ascomycin (*see* specification, page 3, lines 23-24), claim 11 as presently amended provides the necessary antecedent basis for this term with respect to claim 17. Claims 20 and 22 have been canceled from the application, and the rejection of these claims is therefore moot. In view of the foregoing, it is respectfully requested that the rejection of Claim 17 under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn.

Claims 21 and 22 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for ultimately depending from non-elected claim 14. Claims 21 and 22 are presently canceled from the application. The rejection is therefore moot.

Claims 11, 19 and 21 have been rejected under 35 U.S.C. § 102 (b) as allegedly anticipated by U.S. Patent 5,359,060 to Hauske.

Hauske has been cited for teaching purification of FK520 by counter current distribution using 10:1 heptane:acetonitrile solvent system. In making the rejection, the Examiner has directed Applicants' attention to column 9, lines 54-59 and column 10, lines 40-43.

Column 9, lines 54-62, provides:

> The concentrate was subjected to four tube counter current distribution in 20 liter carbuoys using 10 liter top layer and 1 litter bottom layer per carbuoy of a heptane/acetonitrile 10/1 system. The active bottom layers were collected, combined and concentrated. The material was further purified via filtration through Florisil (washing with hexane, hexane/methylene chloride and methylene chloride, successively, with a gradual increase in methylene chloride).

It is noted that in making the rejection, the Examiner included Claim 11 as part of the set of claims being rejected, yet concluded that only Claims 19 and 21 were taught.

For the sake of completeness, this response assumes that the Examiner's conclusion reached in this rejection also pertains to Claim 11.

In contrast to Hauske, claim 11 of the present application recites that heptane and acetone or heptane and isopropanol is used in the lighter phase of the counter current separation and water and acetone or water and isopropanol is used for the heavier phase. Nowhere in Hauske is there a teaching for the use of two different solvent mixtures for the two different phases, wherein the lighter phase flows counter to the heavier phase. Moreover, nowhere in the reference is the mixture of these solvents even mentioned. Claim 11 is therefore distinguished from the teaching provided by Hauske. Withdrawal of the rejection of claim 11 under 35 U.S.C. § 102 (b) is therefore warranted. Claim 19 and 21 have been canceled from the application and the rejection as pertains to these two claims is therefore moot.

Clams 19-22 have been rejected under 35 U.S.C.§ 102(b) as allegedly anticipated by EP 652,219 A1 (hereinafter "Gletsos"). Gletsos teaches the purification of both Rapamycin and FK506 by extraction. In contrast, claims 19-22 recite rapamycins and ascomycins produced by counter current separation. Citing the MPEP 2113 and *In re Thorpe*, 777 F.2d 695, 698, 227 USPQ 964,966 (Fed. Cir. 1985), the Examiner has asserted the proposition that patentability of a product does not depend on its method of production, but rather non-obvious differences in the product. Applicants respectfully submit that claims 19 to 22 are directed to products that are novel and non-obvious over Gletsos. In order to advance prosecution of this application however, and not in any way acquiescing to the position of the Examiner, claims 19-22 have been cancelled without

prejudice. Applicants reserve the right to file one or more divisional or continuation applications directed to the subject matter of the canceled claims.

Claims 19-22 have been rejected under 35 U.S.C. § 102 (e) as allegedly anticipated by U.S. Patent 5,616,595 to Chu et al. Since claims 19-22 have been canceled from the application, the rejection is now moot.

Claims 19-22 have also been rejected under 35 U.S.C. § 102 (b) as allegedly anticipated by EP 427680 A1 to Baumann. The rejection of claims 19-22 is moot since these claims have been canceled from the application.

Claims 19-22 have been rejected under 35 U.S.C. § 102 (e) as allegedly anticipated by U.S. Patent No. 5,665,772 to Cottens et al. As these claims have been cancelled from the application, the rejection is moot.

Claims 19 and 21 have been rejected under the judicially created doctrine of obviousness-type double patenting as allegedly unpatentable over claim 1 of U.S. Patent No. 6, 706,727. The rejection is now moot since claims 19 and 21 have been cancelled from the application.

Applicants acknowledge the Examiner's finding that Claims 12, 13 and 17 contain allowable subject matter and would be allowed if rewritten to overcome the rejection(s) under 35 U.S.C. § 112, 2nd paragraph.

Accordingly, in view of the foregoing remarks and amendments, the present

application is believed to be in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

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Identification of genes coding enzymes for ascomycin tetra-hydropyranose ring formation

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Abstract. Macrocyclic polyketides have generated great interest in biosynthetic chemistry because of the structural complexity and medicinal activities. The synthetic genes consist of the number and type of active sites of modular polyketide synthases. The cosmid library - prepared with the ascomycin (an antibiotics with immunosuppressive activity) - producer, *Streptomyces sp.* AA6554 genome was screened with an ascomycin ketosynthase gene probe, and one and a half modules were isolated. Database analysis shows that one of the modules consists of the genes coding a series of enzymes for the tetra-hydropyranose ring synthesis.

Introduction

Polyketides are natural products identified in various species and are especially abundant in fungi and actinomyces. Genetic analysis of polyketide genes separated them into two classes (1). One of the class consists of macrolides which provide an excellent source to the pharmaceutical drugs. Ascomycin, C-21 ethyl analog of FK506 (2) is a 23-member macrolide (3). Ascomycin is also known as immunomycin and FK520. FK506 and rapamycin consisting of similar structures to that of ascomycin (Fig. 1) have potent immunosuppressive properties to inhibit T cell activation both *in vivo* and *in vitro* (3,4). These three compounds contain the pyranose-pipecolinyl region (C1 to C15; Fig. 1) which mimics leucine- (twisted amide) -proline peptide where peptidyl prolyl cis/trans isomerase (PPIase) binds to and causes various biological activities (5).

We identified the genes coding the synthetic enzymes for ascomycin tetra-hydroxypyranose ring, a part of pyranose-pipecolinyl region where it binds to FK506-binding protein. The structure of the module of this gene is different from those of the FK506 synthase gene A, fkbA (6) and rapamycin synthase gene 3, RAPS3 (7,8) which code the synthases for tetra-hydroxypyranose ring for FK506 or rapamycin respectively.

It is important to increase the genetic database of macrocyclic polyketide synthases. Such information will make it possible to manipulate the synthase genes, generate unnatural macrolides and increase the diversity of macrolides dramatically.

Materials and methods

Cloning. Genome DNA was isolated from *Streptomyces sp.* AA6554 and digested with Sau3A partially. The digested DNA was ligated into pWE15 cosmid with *Bam*HI sites at the ends (Stratagene). Ascomycin synthetic gene cluster was isolated by using the ketosynthase (KS) gene as a probe. The ascomycin KS gene was cloned by PCR. The primers for the PCR (5' primer, 5'-TTCGGGATCAGTCCTCG-3'; 3' primer, 5'-AGGATGACGTGGGCGTT-3') were designed to cover the highly conserved region of the KS gene by comparing the sequence of the KS gene for DEBS1 (2) and that for RAPS3 (7). The amplified product (1047 bp) was sequenced, compared with the other KS genes and confirmed to be a KS gene. The cosmid library was screened with the ascomycin KS gene as the probe. One of the positive clones was picked up, fragmented with sonication and subcloned into pUC19 plasmid to be sequenced.

Sequence analysis. The DNA sequencing was done on double-strand DNA templates with dideoxy method using an automatic sequencer (Applied Biosystems, Model 377 sequencer). The random sequences were compiled and the assembly was performed with the Applied Biosystem Auto Assembler (ABI). The deduced protein sequences were compared with sequences in the GenBank database using the BLAST program (9) and the alignments were performed using the PILEUP and CLASTW program (10).

Results and Discussion

Streptomyces sp. AA6554, high producer of ascomycin was newly isolated from soil. We speculated that the biosynthetic

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Key words: polyketide, ascomycin, tetra-hydropyranose ring

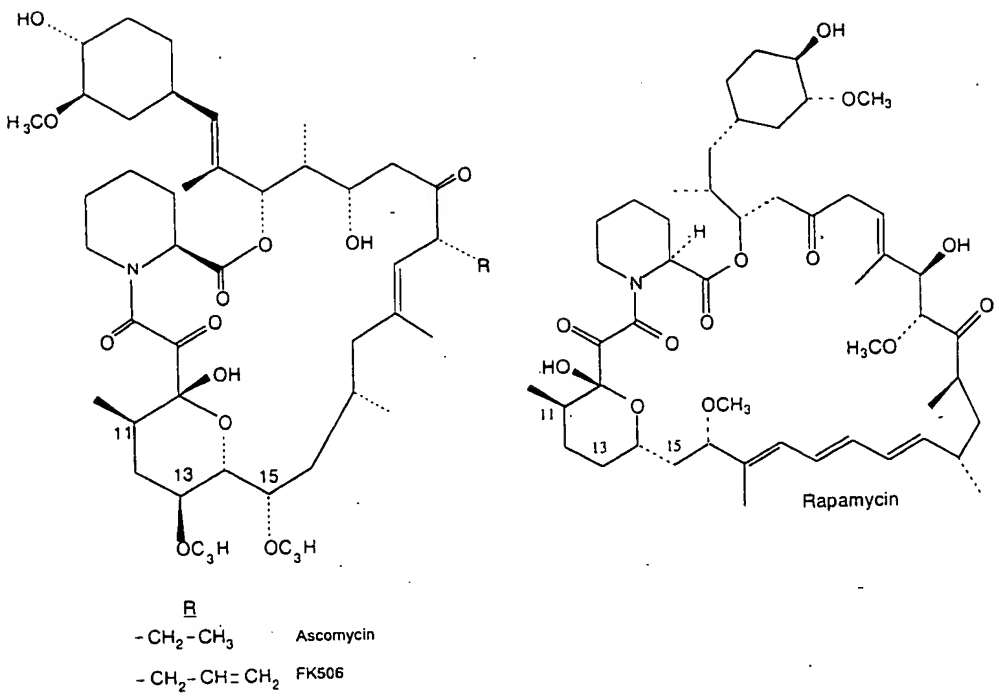

Figure 1. Structure of ascomycin. FK506 and rapamycin. Ascomycin, C-21 ethyl analog of FK506 is a 23-member macrolide. Ascomycin is also known as immunomycin and FK520.

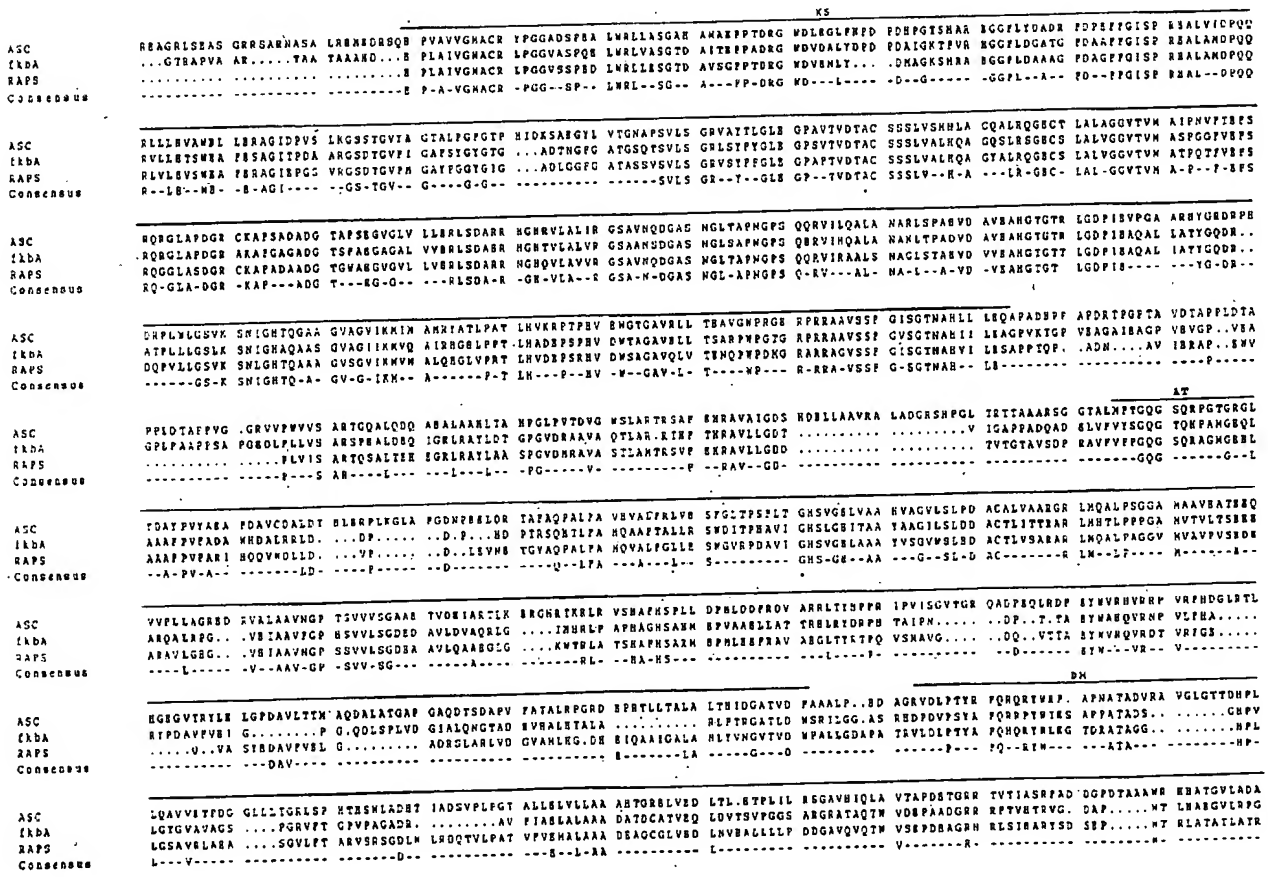

Figure 2. Continued on the next page.

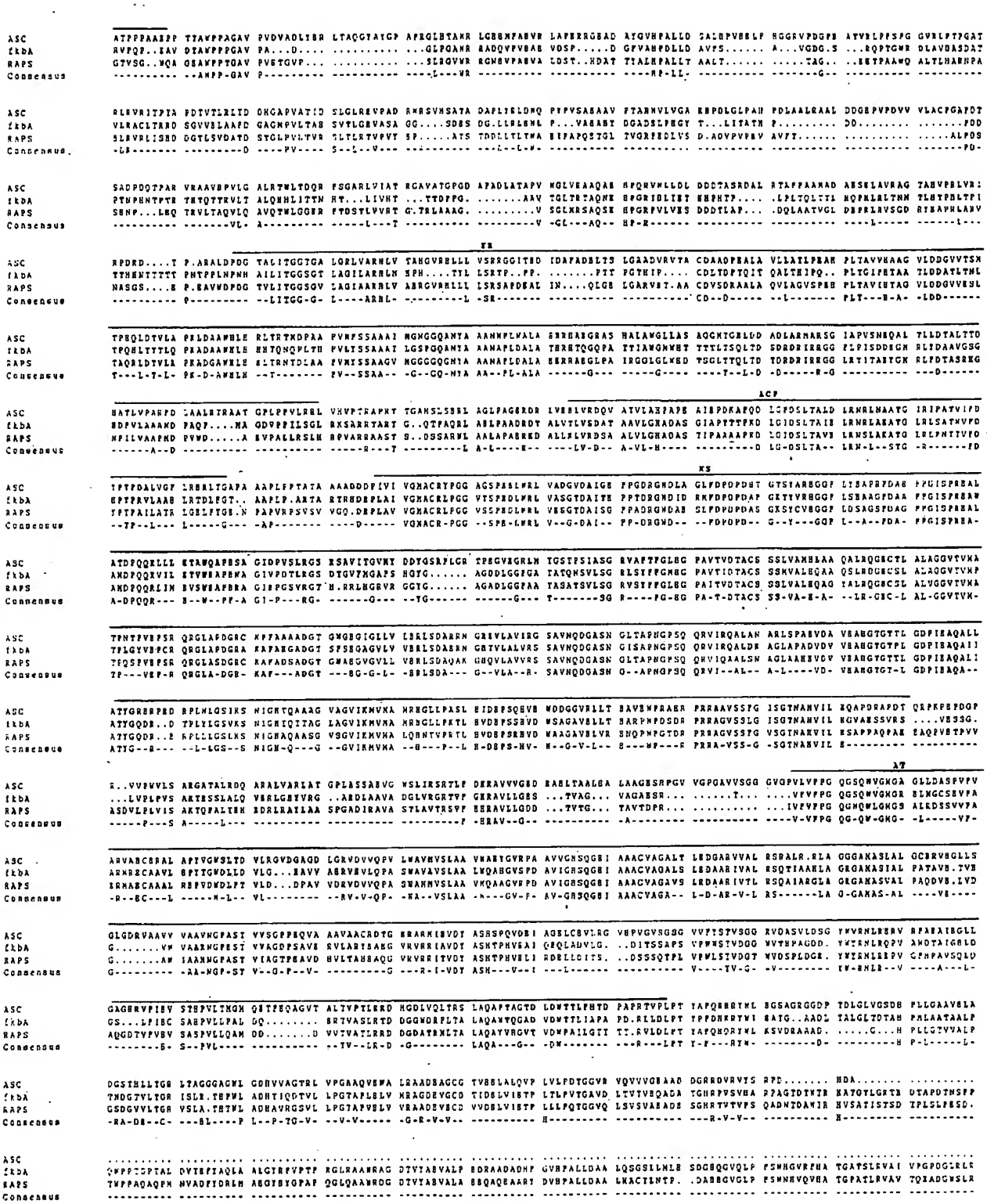

Figure 2. Comparison of the deduced amino acid sequence of ascomycin synthase gene with those of fkbA and RAPS3. The consensus sequence is shown under their sequences. One complete module containing a KS, an acyltransferase (AT), a dehydratase (DH), an enoyl reductase (ER) and an acyl carrier protein (ACP), and a part of the module containing a KS and an AT were identified.

genes for ascomycin consist of a complex of modules like other macrolides, such as erythromycin (2,6), rapamycin (7,8) and FK506 (11). The sequence of ß-ketoacyl synthase (KS) gene of ascomycin synthetic genes is similar to those of the other macrolide synthetic genes. The amino acid sequence of KS of 6-deoxyerythronolide B synthase (DEBS) (2) and that

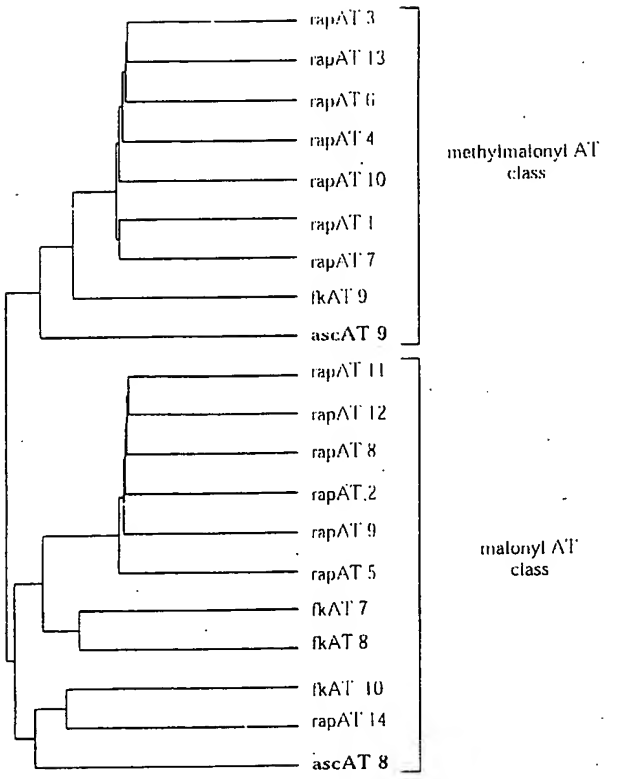

Figure 3. The PILEUP analysis of acyltransferase (AT) domains in these modules. The analysis suggested that the substrates for the identified ATs are acetate (ascAT8) and propionate (ascAT9) respectively.

of rapamycin synthase (RAPS) (7,8) showed high homology to each other, but they have little homology to those for aromatic polyketide synthetic genes (1). The DEBS KS gene probe hybridized to RAPS KS gene or vise versa but neither KS gene probe hybridized to the KS genes of aromatic polyketide synthases (data not shown). We compared the sequences of DEBS1 and RAPS3, and synthesized the PCR primers covering the high homologous sequences around the active sites of the KS genes. DNA fragment (1.1 kb) containing KS active sites was amplified with PCR using the primers. The sequence of the fragment showed high homology to those of DEBS1 and RAPS3 KS genes, especially the active site regions of the KS genes are very well conserved. These results showed that the isolated fragment is an ascomycin KS gene, so we used it as the probe to screen the ascomycin synthase genes.

Fifty-four positive clones were isolated from the cosmid library. Southern-blot analysis of genomic DNA from ascomycin producer cells suggested that the total size of the ascomycin synthase genes is included in 82 kb. We chose the number 44 clone carrying 8 kb insert and determined the sequence completely. Comparison of the deduced amino acid sequence of the clone with the proteins in the database revealed that it contained the consensus active sites of fatty acid synthases and polyketide synthases (12). One complete module containing a KS, an acyltransferase (AT), a dehydratase (DH), an enoyl reductase (ER) and an acyl carrier protein (ACP), and a part of the module containing a KS and an AT were identified (Fig. 2). The amino acid sequence of these enzyme domains corresponded to the module 12 and 13 of RAPS3 and module 8 and 9 of fkbA.

The sequence of KS domain was conserved well between the macrolide synthases. But other enzymes, AT, DH, ER and ACP showed less homology to each other. The KR domain contains a potential NAD(P)H binding motif, GXGXXAXX-XA (8,12). The KR domains of the modules indicated that the KR is active because it contains LGDSL motif where 4'-phosphopantetheine attaches (12). The PILEUP analysis of AT domains of these modules showed that the substrates are acetate and propionate respectively (Fig. 3). The main structure of ascomycin is speculated to be synthesized with poly-merization of acetate and propionate in the following order; shikimic acid - propionate - propionate - acetate - butylate - propionate - acetate - acetate - propionate - acetate - pipecolic acid. This sequential arrangement exists only at the C10 to C13 position of ascomycin, which gives a pyranose-ring, in other words tetrahydropyran (Fig. 1). Taken together, we concluded that these modules correspond to modules 8 and 9. DH was identified in the module 8 (Fig. 2), although DH activity in this module is not required for ascomycin biosynthesis. The motif, HxxxGxxxxP is speculated essential for DH activity. The motif of this DH has the mutation of the Gly to Asp (12). DHs are sometimes inactivated by mutation or deletion of amino acids at the active sites, for example the DH of fkbA module 8 and RAPS module 2, 5, 11 and 12 (6-8) contain a five amino acid deletion in the active sites. So the mutated DH in this module is probably an inactive one.

In conclusion, we cloned a part of ascomycin synthetic genes, which code the enzymes for the ascomycin tetra-hydropyranose ring formation.

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